RESERCA
MEMBER OF SWEDISH TOBACCO

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ANALYTICAL METHOD FOR NICOTINE AND COTININE IN SALIVA

SUMMARY: Nicotine and cotinine are extracted from alkalized saliva by use of methylene chloride. The two components are quantified by use of gas chromatography using a N-selective detector. Structurally similar analogues are used for internal standards.

EQUIPMENT:

GC system: VARIAN model 3700, 3400, 3500, equipped with splitless injectors and nitrogen sensitive detectors.

Capillary columns: SP-1000 (Nanocoat, Finland) 25 m x 0.32 mm i.d. 0.25 um coating.

Integrators: Shimadzu C-R3A, C-R5A and HP 3388A

Sample concentrators: Pierce Reacti-Therm

Centrifuge: Heraeus Christ cooled model Minifuge T, equipped with rotor 1B. Radius 18.4 cm.

Shaker: Desaga Shaker

Test tubes: 5 ml Becton Dickinson Vacutainer tubes with 0.5 ml citrate solution, equipped with Teflon septum in screw caps (Labora).
10 ml centrifuge tubes with Teflon septum in screw caps.

Sample vials: 2 ml septum vials (VARIAN) with conical bottom (made in the lab)
2 ml brown septum vials

Pipettes: 5-1000 ul Automatic dispensing pipettes model Pipetman (Gilson) and Transferpette (Brand). Pasteur pipettes.

GC syringes: 1.0 ul Hamilton 7001.

Reagents: Sodium hydroxide (Pronalys, M&B). 5 M solution shaken with methylene chloride before use.
Methylene chloride (UV spectroscopy quality, Fluka AG)
Ethanol 99.9% (Spectroscopy quality, Kemetyl)

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STANDARDS AND CALIBRATION SOLUTIONS:

(-)-Nicotine: Vacuum distilled and stored under nitrogen in brown ampoules at -80°C Standards are made by weighing 100 mg nicotine into a 100 ml measuring glass (brown) and dissolving in EtOH. This stock solution is then diluted 1:10, 1:100 and 1:1000.

(-)-Cotinine: Our own synthesis according to McKennis. Double distilled under vacuum. Chrystals are stored in a dark container at -20°C. Stock solution and dilution series as for nicotine.

Internal standards: *N-methylanabasine* (for the quantification of nicotine) and *N-ethylnorcotinine* (for the quantification of cotinine) are syntizised according to McKennis and purified by flash and column chromatography.
Stock solutions are prepared as for nicotine and cotinine. From the stock solutions are prepared combined internal standard solutions, the concentrations depending on level of analyts expected.

Calibration solutions: 1:1 solution containing equal amounts of nicotine, cotinine, methylanabasine and ethylnorcotinine. 0.5 ml of solutions having the concentration 1.0 mg/ml are dispensed into a 100 ml measuring glass and diluted to volume with EtOH. This gives 5 ug/ml of each analyte.The solution is kept in brown septum vials at -20°C.
For low concentrations this calibration solution is diluted 1:10.

SAMPLE PREPARATIONS:

Saliva is collected in 5 or 10 ml test tubes and is stored at -20°C.

WORK UP OF SAMPLES:

The frozen saliva samples are thawed and centrifuged for 10 minutes at 3000 rpm(1850 g) and at a temperature of 10°C.
0.5-1-0 ml clear supernatant is collected and to that is added internal standard solutions. Normally 200 ng of methylanabasine and ethylnorcotinine is acceptable level for work with smokers. To the sample is added 1ml 5M NaOH and 2 ml methylene chloride.
The sample is shaken for 5 minutes and then centrifuged for 20 minutes at 3000 rpm and a temperature of -5°C.
The supernatant water phase is removed with a Pasteur pipette connected to a water ejector. The organic layer is decanted into sample vials that are stored at -20°C until used for analysis. The content in the vial is then concentrated to approximately half the volume and then EtOH is added and the content is further concentrated down to 10-50 ul.

GC PARAMETERS:

Carrier gas: Helium. Flow rate 2.4 ml/min
Detector gas:H_2. Flow rate 3.3 ml/min. Make up He at 27 ml/min. Air at 240 ml/min.
Injector temperature: 290 °C

Detector temperature: 300 °C
Injection volume: 0.1-0.3 ul

Oven temperature: Initial temp 135 °C. Initial time 10 minutes
Temp programming rate 30 degrees per minute
Final temp 220 oC. Final time 15 min.

Retention times: Nicotine 5.1 minutes. Methylanabasine 7.7 minutes
Cotinine 16.9 minutes. Ethylnorcotinine 17.3 minutes

CALCULATIONS:

Relevant retention times, response factors and amounts of internal standards are programmed into the integrator for calculation of amounts of analyte.
Response factors are found by dividing the area for the Internal Standard by the area for the analyte.

VALIDATION:

Calibration: The GC performance is evaluated by injecting Calibration Solution and responce factors and retention times are controlled in the beginning and at the end of each series of samples and furthermore at least once a month.
The total method is checked by using samples of saliva pooled from a number of tobacco users.

Linearity: The linearity of the GC responce has been validated by using standard solution and constructing a standard graph. Saliva from non-tobacco users (0-saliva) is spiked with nicotine in concentrations 1-10 ng/ml and cotinine 1-10 ng/ ml and 5-500 ng/ml.

Sensitivity:

Detection limits are 5 pg for both nicotine and cotinine at a S/N ratio of 2.
Minimum detectable concentration is 0.5 ng/ml

Reproducibility:

The reproducibility has been verified by the use of standard solution graphs.
Coefficients of variation for both variability during the same day and during different days are given in the table on next page.

Yield of extraction:Nicotine and cotinine can be quantitatively extracted from saliva by the used method.

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